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PRIMARY RESEARCH PAPER

Ethanol preservation effects on stable carbon, nitrogen and hydrogen isotopes in the freshwater pearl mussel

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Abstract Chemical preservatives can alter stable isotope ratios in animal tissues. The effects of preservation on $\delta^{13}C$ and $\delta^{15}N$ values have been investigated in a variety of species, but not on $\delta^{2}H$ values or on the freshwater pearl mussel (FPM, *Margaritifera margaritifera*) tissues. We evaluated the effect of ethanol preservation (unpreserved vs preserved tissues) over 6 months on the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values of FPM foot and gonad tissues. Ethanol preservation significantly increased $\delta^{13}C$ values (foot 0.4 ‰; gonad 0.3 ‰), whereas it did not significantly affect $\delta^{15}N$ values (foot 0.2 ‰; gonad −0.1 ‰). The positive effect of ethanol preservation on $\delta^{2}H$ values (foot 7.1 ‰; gonad 14.5 ‰) and the negative effect on C:N ratios (foot −0.1; gonad −0.5) depended on the tissue type, with larger effects found on the lipid-rich gonad. Overall, ethanol preservation affected $\delta^{2}H$ values more than the $\delta^{13}C$, $\delta^{15}N$ or C:N ratios of FPM tissues. After 1 month of preservation, the isotope values remained rather stable, and significant changes were only observed in $\delta^{15}N$ values. The results imply that ethanol-preserved FPM samples can be used if potential shifts in isotopic and elemental ratios are accounted for prior running mixing models for estimating dietary proportions.

Keywords C:N ratio · Deuterium · *Margaritifera margaritifera* · Stable isotope analysis · Ethanol storage · Lipids

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Introduction

The use of stable isotope analysis (SIA), such as the ratios of naturally occurring stable isotopes of carbon ($^{13}C/^{12}C$; hereafter denoted as $\delta^{13}C$), nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$) and hydrogen ($^{2}H/^{1}H$; $\delta^{2}H$), has improved our understanding of how aquatic ecosystems work (Fry, 2006). Stable isotope ratios are routinely used to study the structure and function of food webs and the energy sources of organisms within (Umbricht et al., 2018). Because primary producers vary in their isotopic composition, $\delta^{13}C$ is generally used to determine the origins of carbon in food webs or to identify feeding areas (DeNiro & Epstein, 1978). As organisms are generally enriched in ^{15}N relative to their diet, they show an increase in $\delta^{15}N$ values with each trophic step in the food web. $\delta^{15}N$ can also be used to assess nitrogen sources (Deniro & Epstein, 1981). $\delta^{2}H$ values have been applied to differentiate terrestrial from aquatic inputs because $\delta^{2}H$ varies between terrestrial and aquatic primary producers (Doucett et al., 2007) or to study dispersal of animals (Hobson & Wassenaar, 2008). C:N ratios in

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animals, including aquatic species, represent the overall macronutrient content in any given tissue and have been used to estimate lipid content of the tissue (Post et al., 2007).

Despite SIA being used successfully in ecological studies for over 3 decades, there are still many unknowns about the effects of sample treatment on isotope values (Lau et al., 2012). For SIA, fresh samples are preferred, but logistics often require the preservation of organisms for later analyses (Jesus et al., 2015). Preserved samples are also often found from museums or research institutes and SIA of such archived tissue samples may provide the only record about long-term ecosystem changes (Syväranta et al., 2008). Stable isotope values measured in animal tissues represent values generated by their diet during a given period (DeNiro & Epstein, 1978). However, many factors, including the preservation method and time, can affect the stable isotope ratios in animal tissues and therefore also the results of isotope mixing models used for estimation of consumer diets (Javornik et al., 2019). Since the isotope ratios are sensitive to even subtle variations, meticulous care must be taken to preserve samples (Yurkowski et al., 2017).

Numerous studies have examined preservation effects on stable isotope ratios of carbon and nitrogen, with inconsistent results across taxa, environments (e.g., marine vs freshwater), tissue types, preservation methods and duration of preservation (Vizza et al., 2013; Kiszka et al., 2014; Stallings et al., 2015; Hogsden & McHugh, 2017). Chemical preservatives can alter stable isotope ratios through a variety of mechani2004sms: they can either add carbon or nitrogen to tissues or they can leach macromolecules consisting of these elements (Sarakinos et al., 2002; Sweeting et al., ; Barrow et al., 2008; Ruiz-Cooley et al., 2011). Ethanol is often used as a preservative to store animal tissues. Since ethanol does not contain nitrogen, it cannot add nitrogen to samples, but it may affect stable isotope ratios by breaking bonds with nitrogen atoms in tissues (Hetherington et al., 2019). Some studies reported that ethanol storage did not affect the δ^{15}N values in sea stars, freshwater invertebrates and fish fin (Vizza et al., 2013; Hogsden & McHugh, 2017; Le Bourg et al., 2020). In contrast, other studies indicate that ethanol can increase δ^{15}N values in bivalves and ray fishes (Syväranta et al., 2011; Burgess & Bennett, 2017; Umbricht et al., 2018) but decrease δ^{15}N values in some other fishes (Olin et al., 2014). Carbon content of tissues and their δ^{13}C values may also be affected by ethanol but the magnitude and direction of changes varies among studies. For instance, previous studies of marine and freshwater bivalves, invertebrates, fishes and mammals indicate that ethanol preservation can either have no effect (Lau et al., 2012; Kiszka et al., 2014; Burgess & Bennett, 2017) or lead to higher δ^{13}C values of consumer tissues (Syväranta et al., 2011; Stallings et al., 2015; Hogsden & McHugh, 2017; Umbricht et al., 2018). Lipids are depleted in ^{13}C and ^{2}H as compared to proteins and carbohydrates (DeNiro & Epstein, 1978; Hobson et al., 1999). Thus, if ethanol extracts lipids from samples, a decrease in C:N ratio as well as an increase in the δ^{13}C and δ^{2}H values of preserved samples would be expected.

Freshwater bivalves are commonly used in biomonitoring (Farris & Van Hassel, 2007). They can be important components in freshwater food webs, provide valuable ecosystem services and have a large impact on ecosystem function, including nutrient cycling (Vaughn et al., 2008; Atkinson & Vaughn, 2014; Vaughn, 2018). Studies on the effect of ethanol preservation on stable isotope values of freshwater bivalves, Asiatic clams apart (Sarakinos et al., 2002; Syväranta et al., 2011), are practically non-existent. However, ethanol-preserved and archived samples of endangered freshwater mussels are indispensable sources of material for isotope analysis because they can be used to reconstruct historical energy and nutrient sources. Moreover, to the best of our knowledge, no studies have examined the effect of chemical preservation on δ^{2}H values of animal tissues. Hydrogen stable isotopes are increasingly used to study aquatic food webs and animal migrations, reinforcing the need to understand how different storage and laboratory preparation methods affect δ^{2}H values (Hobson & Wassenaar, 2008; Soto et al., 2013).

This study investigates the effect of ethanol preservation and its duration on δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios of the endangered freshwater pearl mussel [FPM, *Margaritifera margaritifera* (Linnaeus, 1758)]. The obtained results will allow subsequent correction for potential preservation-induced changes in FPM isotope values prior to comparing or using data from preserved and non-preserved samples in isotope mixing models. We prepared FPM foot and gonad tissues for SIA to evaluate if the ethanol

preservation effects varied among tissue types, with lipid-rich gonads expected to show larger changes. Specifically, we addressed the following questions: (1) How do isotope values and C:N ratios differ between unpreserved and ethanol-preserved tissues? (2) How does preservation time affect differences in isotope values and C:N ratios in FPM tissues? Based on previous studies, we hypothesized that ethanol preservation may increase $\delta^{13}C$ and $\delta^{2}H$ values, decrease C:N ratios of preserved samples and have no or minor effect on $\delta^{15}N$ values. Further, we assumed that ethanol preservation would have a larger effect on the lipid-rich gonads (Jokela et al., 1993) than on the foot tissue of FPM. Moreover, we predicted that shifts in isotope values should increase with preservation time.

Materials and methods

Mussel collection and preparation

Twenty-four FPM individuals were collected from eight rivers, three individuals per river, in Northern Finland (64–68 °N, 27–29 °E) during August–September 2020. FPMs were collected under permission (KAIELY/296/2019 and 357/2019, LAPELY/1929/2019 and 2252/2019, POPELY/1276/2019 and 1490/2019) granted from the Centers for Economic Development, Transport and the Environment of Kainuu, Lapland and North Osthrobothnia, Finland, respectively. Upon collection, the mussels were packed in individual plastic bags, stored immediately in ice, transported to the laboratory and kept in a cool box under ice until dissection. Mussels were dissected and tissues (foot and gonad) were extracted. In previous studies (Syväranta et al., 2011; Xu et al., 2011; Liu et al., 2013), the ethanol preservation effects on $\delta^{13}C$ and $\delta^{15}N$ values have been shown to stabilize after 6 months preservation. Therefore, we decided to conduct this experiment for only 6 months. To examine the effects of ethanol preservation and preservation time (1, 2, 4, and 6 months) on $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values and C:N ratio, the foot and gonad samples dissected from each FPM individual were split in two parts; one stored at − 18 °C ('unpreserved tissue'/time 0) overnight prior to freeze-drying, and another stored in 99.5% ethanol and kept at room temperature (~ 18 °C) ('preserved'). The preserved samples of FPM foot and gonad tissues were removed from ethanol and dissected for subsamples after 1, 2, 4 and 6 months, rinsed with ultra-pure water and then freeze-dried. The unpreserved and preserved freeze-dried samples were ground to a fine powder with a mortar and pestle. Prior to final SIA, 0.500–0.700 mg of sample was weighed into a tin cup for $\delta^{13}C$ and $\delta^{15}N$ analyses and 0.350 mg of sample into a silver cup for $\delta^{2}H$ analysis. Prior to folding, the silver cups were stored open in laboratory atmosphere with laboratory standards for at least five days to allow hydrogen exchange between the samples and laboratory air (Wassenaar & Hobson, 2003). To examine the effect of preservation time, the relative differences (Δ) in isotope values of preserved samples (time points 1–6 months) to the values of unpreserved samples (time 0) were calculated.

Stable isotope analysis

We measured $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values and C:N ratios at the Stable Isotope Laboratory at the University of Jyväskylä, Finland. We report stable isotope ratios in δ notation relative to Vienna Pee Dee belemnite (VPDB), atmospheric nitrogen (N_2) and Vienna Standard Mean Ocean Water (VSMOW) for carbon, nitrogen, and hydrogen, respectively. $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios (by mass) were measured by continuous-flow stable isotope ratio mass spectrometer (CF-SIRMS) coupled with a FlashEA 1112 elemental analyzer (Thermo Electron Corporation, Waltham, MA, U.S.A.). $\delta^{2}H$ values were measured using an Isoprime 100 CF-SIRMS (Isoprime Ltd, Stockport, U.K.) coupled with Elementar vario PYRO cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). In each run, multiple samples of two reference materials (caribou hoof [CBS], kudu horn [KHS]) were analyzed relative to VSMOW (Standard Mean Ocean Water) (Soto et al., 2017). Carbon, nitrogen and hydrogen stable isotope ratios are expressed as per mil (‰) and calculated following the equation:

$$\delta X = \left[\left(\frac{R_{sample}}{R_{reference}} \right) - 1 \right] \times 1000 \quad (1)$$

where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{2}H/^{1}H$ ratios for the sample and international standards, respectively. X represents the heavy isotopes of ^{13}C, ^{15}N or ^{2}H. Positive δ values indicate that the sample is isotopically enriched, meaning it

contains a greater proportion of the heavy stable isotope (^{13}C, ^{15}N or ^{2}H). The standard deviations of the delta-values of replicate reference materials in each run were always less than 0.14 ‰ for $\delta^{13}C$, 0.24 ‰ for $\delta^{15}N$ and 2.41 ‰ for $\delta^{2}H$.

Statistical analyses

We used Generalized Linear Mixed Effect Models (GLMM) in the statistical software R (version 4.1.2; 2022-09-06; R Core Team, 2022) to test for the effects of ethanol preservation (unpreserved samples at time point 0 and preserved samples at time point 6; hereafter *Preservation*) and preservation time (preservation for 1, 2, 4, and 6 months; hereafter *Time*) on the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values and C:N ratios of FPM foot and gonad tissues (hereafter *Tissue*). For the GLMMs, we used *lme* function with default settings in the *nlme* package (Pinheiro et al., 2021) and set the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values and C:N ratios as the response variables and ethanol preservation (*Preservation*), preservation time (*Time*), tissue type (*Tissue*) and the *Preservation* x *Tissue* and *Time* x *Tissue* two-way interactions as the explanatory variables and FPM individual as the random factor (random = ~ 1|Individual). In the case of significant differences ($P < 0.05$), Tukey-tests were used for pairwise post hoc comparisons to compare the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values and C:N ratios between different time points, using the emmeans package in R (Lenth et al., 2022).

Table 1 Results of Generalized Linear Mixed Effect Models (GLMM) predicting $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values and C:N ratios of foot and gonad tissues of freshwater pearl mussel, with *Preservation* [unpreserved (t = 0) and ethanol-preserved samples (t = 6)], *Tissue* (foot and gonad) and the two-way *Preservation* x *Tissue* interaction as the explanatory variable

	NumDF	denDF	*F* value	*P* value
$\delta^{13}C$				
Intercept	1	67	9164.78	< 0.001
Preservation	1	67	33.64	**< 0.001**
Tissue	1	67	230.35	**< 0.001**
Preservation × Tissue	1	67	0.19	0.666
$\delta^{15}N$				
Intercept	1	67	285.41	< 0.001
Preservation	1	67	1.093	0.300
Tissue	1	67	97.204	**< 0.001**
Preservation × Tissue	1	67	2.253	0.138
$\delta^{2}H$				
Intercept	1	67	13,723.47	< 0.001
Preservation	1	67	111.69	**< 0.001**
Tissue	1	67	46.65	**< 0.001**
Preservation × Tissue	1	67	13.87	**0.001**
C:N ratio				
Intercept	1	55	3770.063	< 0.001
Preservation	1	55	16.27	**< 0.001**
Tissue	1	55	197.05	**< 0.001**
Preservation × Tissue	1	55	7.27	**0.009**

Significant differences ($P < 0.05$) are highlighted in bold

Results

As indicated by the significant two-way interactions (Table 1), the positive effect of ethanol preservation on $\delta^{2}H$ values and the negative effect on C:N ratios depended on the tissue type, with the effects being larger in the lipid-rich gonad than in the foot tissue (Fig. 1). However, no significant *Preservation x Tissue* interaction effects were detected for $\delta^{13}C$ or $\delta^{15}N$, as gonads were consistently depleted in both ^{13}C and ^{15}N compared to foot tissue, and the significant positive main effect of *Preservation* on $\delta^{13}C$ was comparable in both tissue types (i.e., 0.3–0.4 ‰; Tables 1, 2, Fig. 1).

Ethanol preservation increased $\delta^{13}C$ values in both tissues but with a slightly higher magnitude in foot (0.4 ‰) than in gonad (0.3 ‰; Table 2). In contrast, preservation had minor and inconsistent effects on $\delta^{15}N$, with the shift in $\delta^{15}N$ ranging from −0.1 ‰ to 0.2 ‰ in gonad and foot, respectively (Table 2). Both tissues showed elevated $\delta^{2}H$ values in preserved samples as compared to the unpreserved samples by a mean difference of 7.1 ‰ and 14.5 ‰ in foot and gonad (Table 2), respectively. C:N ratios were overall higher in gonad than foot and decreased 0.1 in foot and 0.5 in gonad after 6 months' preservation.

The GLMM results indicated no significant *Time x Tissue* interaction effects for $\Delta\delta^{13}C$, $\Delta\delta^{15}N$, $\Delta\delta^{2}H$ or ΔC:N ratios (Table 3). In contrast, the results show a significant main effect of *Time* on $\Delta\delta^{15}N$, but not for $\Delta\delta^{13}C$, $\Delta\delta^{2}H$ and ΔC:N ratios (Table 3). However, as indicated by the pairwise comparisons,

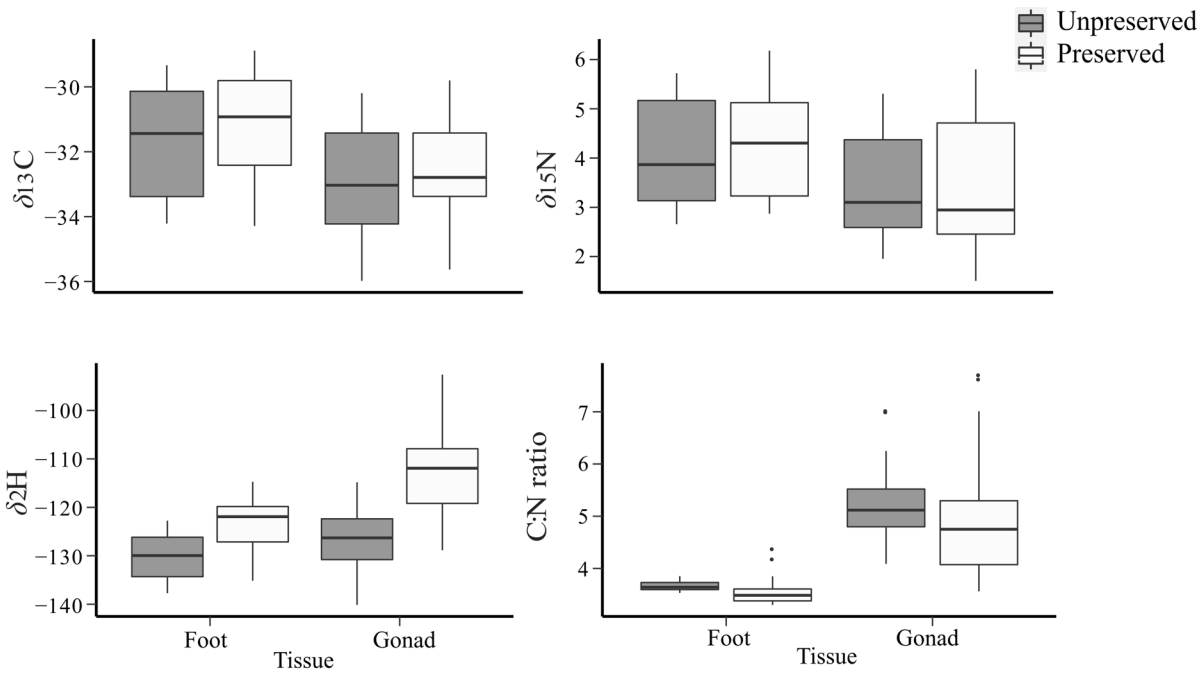

Fig. 1 Boxplots showing $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values (‰) and C:N ratios of unpreserved (time = 0) and preserved foot and gonad (time = 6) in ethanol of freshwater pearl mussel. The central box spans the interquartile range with the middle line denoting the median and whiskers defining minimum and maximum range

Table 2 Mean ± standard deviation (SD) and the observed minimum (min) and maximum (max) values of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and C:N ratios of unpreserved (time 0) and preserved (time 6) foot and gonad tissues of freshwater pearl mussel

	Unpreserved				Preserved				Differences (Δ)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	mean	SD	min	max
Foot												
$\delta^{13}C$	−31.6	1.7	−34.2	−29.3	−31.2	1.6	−34.3	−28.7	0.4	0.1	−0.7	1.3
$\delta^{15}N$	4.1	1.1	2.7	5.7	4.3	1.1	2.7	6.2	0.2	0.1	−0.3	0.9
$\delta^{2}H$	−130.1	4.8	−137.7	−122.8	−123.0	5.2	−135.1	−101.3	7.1	0.5	−0.8	16.0
C:N ratio	3.7	0.1	3.5	3.9	3.6	0.3	3.3	4.9	−0.1	0.2	−0.4	1.4
Gonad												
$\delta^{13}C$	−32.9	1.8	−36	−30.2	−32.6	1.6	−35.6	−29.2	0.3	0.2	−1.1	2.1
$\delta^{15}N$	3.5	1.1	2.0	5.3	3.4	1.3	1.5	6.0	−0.1	0.2	−0.9	1.0
$\delta^{2}H$	−126.9	6.2	−140.2	114.8	−112.4	9.6	−133.6	−79.8	14.5	3.4	−13.3	27.8
C:N ratio	5.0	0.6	4.1	6.2	4.6	0.6	3.6	6.7	−0.5	0.1	−1.7	1.7

The Δ values represent the differences between unpreserved and preserved samples

the effect of *Time* on $\Delta\delta^{15}N$ was not significant over the preservation period and resulted from the lower $\Delta\delta^{15}N$ values at months 2 (−0.1 ‰) and 6 (−0.2 ‰) (Fig. 2). Finally, gonads showed higher $\Delta\delta^{2}H$ values (13.3 ‰) but lower ΔC:N ratios (0.5) than the FPM foot tissue at month 6 (Fig. 2, Table 3).

Table 3 Results of Generalized Linear Mixed Effect Model (GLMM) repeated measures for $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and C:N ratios of foot and gonad tissues of freshwater pearl mussel preserved in ethanol for different time periods (month 1–6)

	numDF	denDF	*F* value	*P* value
$\delta^{13}C$				
Intercept	1	122	65.88	< 0.001
Time	3	122	0.60	0.614
Tissue	1	122	0.38	0.537
Time × Tissue	3	122	0.35	0.789
$\delta^{15}N$				
Intercept	1	117	36.5	< 0.001
Time	3	117	6.80	**< 0.001**
Tissue	1	117	0.04	0.851
Time × Tissue	3	117	0.54	0.658
$\delta^{2}H$				
Intercept	1	120	182.01	< 0.001
Time	3	120	1.87	0.138
Tissue	1	120	22.67	**< 0.001**
Time × Tissue	3	120	2.30	0.080
C:N ratio				
Intercept	1	118	2770.58	< 0.001
Time	3	118	1.28	0.283
Tissue	1	118	137.57	**< 0.001**
Time × Tissue	3	118	0.32	0.813

Significant differences ($P < 0.05$) between time periods and tissues are highlighted in bold

Discussion

Our results demonstrate that ethanol preservation can affect the $\delta^{13}C$ and $\delta^{2}H$ values and the C:N ratios of FPM, with the effects being particularly evident in the lipid-rich gonad tissues. The shifts in isotope values and C:N ratios were generally minor, with the largest shifts being observed in the $\delta^{2}H$ values (ca. 7–14 ‰ increase).

The reasons for preservation-induced changes in isotope values are not fully understood, but they can be associated with lipid extraction (Syväranta et al., 2008), exchange of heavy and light isotopes between samples and preservatives (Hobson et al., 1997; Edwards et al., 2002), or protein hydrolysis during preservation (Arrington & Winemiller, 2002; Sarakinos et al., 2002; Lecea et al., 2011). Although this study was not designed to explain the causative mechanisms altering the stable isotope values in FPM tissues, most of the shifts in isotope values could be caused either by loss or uptake of materials during preservation. As compared to proteins and carbohydrates, lipids are depleted in ^{13}C and ^{2}H (Park & Epstein, 1961; DeNiro & Epstein, 1978; Hobson et al., 1999). Several studies have shown that the extraction of isotopically lighter lipids from the samples may increase the $\delta^{13}C$ and $\delta^{2}H$ values of an organism and decrease the C:N ratios (DeNiro & Epstein, 1978; McConnaughey & McRoy, 1979; Gloutney & Hobson, 1998; Kiljunen et al., 2006). The effect of ethanol preservation can arise through either hydrolysis of lipids (Hobson et al., 1997; Gloutney & Hobson, 1998; Bosley & Wainright, 1999) or uptake of ethanol into the tissues (Sarakinos et al., 2002). The increase in $\delta^{13}C$ and $\delta^{2}H$ values of preserved foot and gonad tissues may be explained by ethanol-induced lipid extraction as indicated by the decreased C:N ratios (Kaehler & Pakhomov, 2001; Arrington & Winemiller, 2002; Sweeting et al., 2004). C:N ratios in animals, including aquatic species, provide a strong predictor of lipid content within tissues. C:N ratios of > 4 typically indicate lipid-rich whereas C:N ratios of < 4 indicate lipid-poor tissues (Post et al., 2007). In our study, lipid-rich gonads showed lower $\delta^{13}C$ values and higher C:N ratios in both unpreserved and preserved samples as compared to the foot. Surprisingly, $\delta^{2}H$ values showed an opposite pattern with higher $\delta^{2}H$ in lipid-rich gonads, but both tissues were clearly more enriched after 6 months' preservation. ^{2}H-enriched was larger in the gonads, suggesting that lipid-rich tissues are more likely to lose lipids which are depleted in ^{13}C and ^{2}H (Kaehler & Pakhomov, 2001; Sweeting et al., 2004; Carabel & Verísimo, 2009). Our results correspond with previous studies (Stallings et al., 2015; Hogsden & McHugh, 2017; Umbricht et al., 2018; Le Bourg et al., 2020), but contrast with the previously observed ethanol-induced decrease in $\delta^{13}C$ values of a soft-shell clam species (Umbricht et al., 2018). While ethanol is known to alter isotopic values, recent reviews suggest that preservative effects on different tissues, species, and organisms can be variable and inconsistent across studies, taxa and environments (Hogsden & McHugh, 2017).

The differences in $\delta^{13}C$ values between unpreserved and preserved FPM foot and gonad tissues were + 0.4 ‰ and + 0.3 ‰, respectively. This preservation effect was smaller in magnitude than previously reported for preserved fish muscle tissue (i.e.,

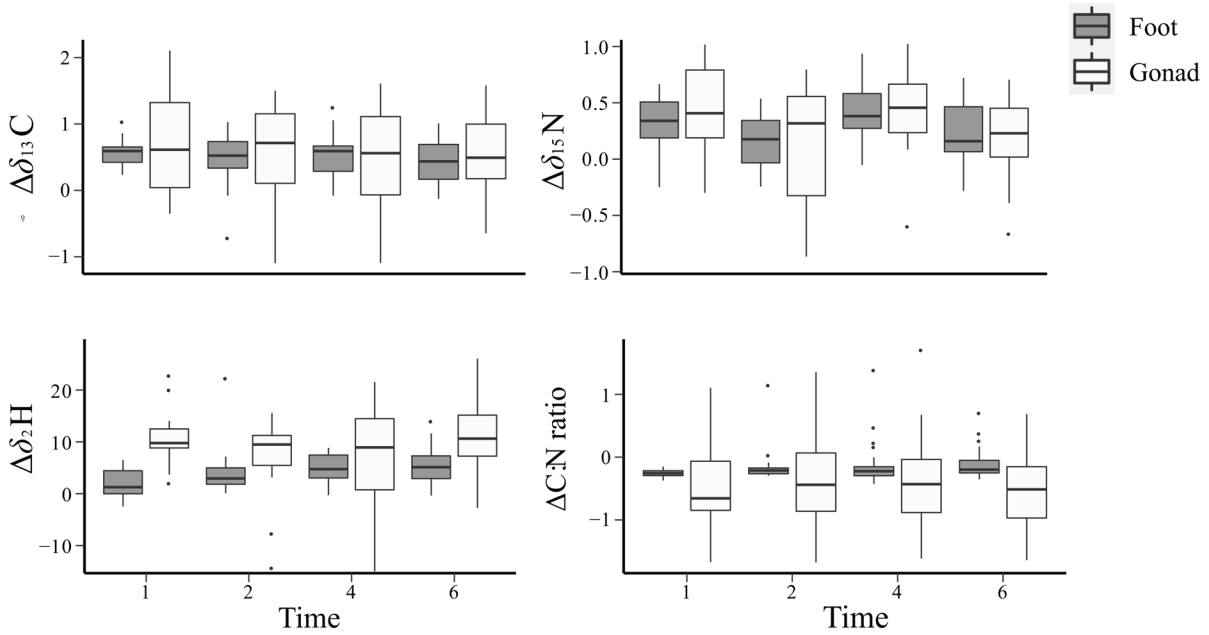

Fig. 2 Boxplots showing the relative differences (Δ) in $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values and C:N ratio of preserved samples (time points 1–6 months) to the values of unpreserved samples (time 0) of freshwater pearl mussel. The central box spans the interquartile range with the middle line representing the median and whiskers defining minimum and maximum range

1.3–1.4 ‰ increase; Vizza et al., 2013), probably due to differences in tissue composition. Similar enrichment was also found in an Asiatic clam [*Corbicula fluminea* (O. F. Müller, 1774)] (Sarakinos et al., 2002) with a higher magnitude of 2.2 ‰, while an increase of 0.8 ‰ was observed in dorsal muscle tissue of Arctic charr [*Salvelinus alpinus* (Linnaeus, 1758)] (Kelly et al., 2006). Thus, the degree of ethanol preservation effect on $\delta^{13}C$ values varies among tissue types, species and preservation times. $\delta^{13}C$ values in animal tissues usually provide information about the consumed food sources (McConnaughey & McRoy, 1979) and the trophic fractionation between the diet and consumer tissues is assumed to be ca. 0–1 ‰ per trophic level (Post, 2002). If preservation causes a shift that exceeds the trophic discrimination factor (TDF), this may cause mis-interpretations in food web studies (DeNiro & Epstein, 1978; Bosley & Wainright, 1999; Bugoni et al., 2008). Thus, the changes in $\delta^{13}C$ observed here (i.e., 0.3–0.4 ‰ enrichment) are comparable to TDFs generally applied in ecological stable isotope studies (Post, 2002).

Ethanol preservation altered the $\delta^{15}N$ values of FPM foot and gonad tissues in different directions. These shifts in $\delta^{15}N$ values of foot (+0.2 ‰) and gonad (– 0.1 ‰) were relatively small when considering the analytical precision here (0.24 ‰ for $\delta^{15}N$) as well as the commonly used TDF values (i.e., 3–4 ‰ increase with each trophic level) (Deniro & Epstein, 1981; Minagawa & Wada, 1984). This suggests that the $\delta^{15}N$ values of preserved FPM tissues can be used in food web studies if unpreserved samples are not available. Findings from our and previous studies (Hobson et al., 1997; Ponsard & Amlou, 1999; Sarakinos et al., 2002; Lau et al., 2012; Olin et al., 2014) demonstrate a minimal effect of ethanol on $\delta^{15}N$ values. However, some other studies have found relatively high increase in $\delta^{15}N$ values, such as 0.7 ‰ for squid muscle tissue (Ruiz-Cooley et al., 2011) and 0.9–1.0 ‰ for Asiatic clams (Sarakinos et al., 2002; Syväranta et al., 2011).

The largest effects of ethanol preservation were observed in $\delta^{2}H$ values, with a significantly higher ^{2}H-enrichment in the lipid-rich gonad (+14.5 ‰) than in the foot tissue (+7.1 ‰). The significant increase of $\delta^{2}H$ in both tissues is likely derived from the loss of ^{2}H-depleted lipids (Hobson et al., 1999). It is possible that structural changes, such as shrinkage or hardening of the tissues, could have caused lipid-release and isotopic enrichment (Singhal et al., 2016;

Panzacchi et al., 2019). The TDFs of δ^2H are yet poorly studied, but there are substantial differences in δ^2H values between terrestrial and aquatic primary producers, with an average difference of ~ 100 ‰ (Doucett et al., 2007). Although the ethanol-induced shifts in δ^2H values of FPM foot and gonad tissues were relatively large as compared to those observed for δ^{13}C and δ^{15}N values, the marked differences between the terrestrial and aquatic isotopic endmembers may allow the use of δ^2H values of ethanol-preserved FPM tissues in isotopic mixing models estimating the reliance of FPM on various food sources (Doucett et al., 2007; Brauns et al., 2021).

Some previous studies demonstrate that preservation time can significantly change stable isotope values (Sweeting et al., 2004; Ruiz-Cooley et al., 2011; McConnaughey & McRoy, 1979), whereas other studies have found no significant effect on δ^{13}C and δ^{15}N values (Gloutney & Hobson, 1998; Barrow et al., 2008). Our results showed significant effects of preservation time only on $\Delta\delta^{15}$N. Our results indicated that the most preservation time-induced changes in $\Delta\delta^{13}$C happened after 1 month of storage for both tissues, although the effect on $\Delta\delta^{13}$C was not significant, as was the case also in some previous studies (Sweeting et al., 2004; Syväranta et al., 2008). Few studies have investigated temporal changes in δ^{13}C and δ^{15}N values of ethanol-preserved samples, but it appears that changes can occur almost instantly (e.g. an increase of δ^{13}C within a week for freshwater clams; (Syväranta et al., 2011). The most striking and temporally stable shifts occurred in the $\Delta\delta^2$H values and ΔC:N ratios of the FPM gonad tissue after 6 months' ethanol preservation. The $\Delta\delta^{13}$C and $\Delta\delta^{15}$N values showed inconsistent and unexplained fluctuations during the preservation experiment but the fluctuations were within the range of analytical error (i.e., 0.14 ‰ for δ^{13}C and 0.24 ‰ for δ^{15}N) and thus may simply represent instrument noise.

Our results indicate that ethanol preservation affects δ^2H values more than δ^{13}C and δ^{15}N values of FPM foot and gonad tissues. Although significant and potentially ecologically relevant shifts were observed for all elements, the effects on δ^{15}N seemed small enough to facilitate the use of ethanol-preserved FPM samples to infer e.g. the trophic position of consumers in food web studies. Considering our findings, we suggest that ethanol should be used with caution and potential isotopic shifts should be accounted for when using preserved bivalves as tracers of environmental changes (Glibert et al., 2018). While our findings are encouraging for samples preserved for short periods, more research is needed to determine whether archived samples of endangered and long-lived FPM can be used to evaluate long-term changes in freshwater ecosystems (Schöne, 2013; Fritts et al., 2017).

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Author contributions MH, JT, and MK all conceived of and designed the experiments, performed the experiments, and collected the data. MH, APE and MK analyzed the data. MH, JT, APE, and MK contributed to writing the manuscript.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Data availability All the data and materials will be provided, if requested.

Code availability Not applicable.

Ethical approval Applicable.

Consent to participate All authors have given their consent to participate.

Consent for publication All authors have given their consent for publication.

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